

# Mitofusins 1/2 and ERR $\alpha$ expression are increased in human skeletal muscle after physical exercise

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Mitochondrial impairment is hypothesized to contribute to the pathogenesis of insulin resistance. Mitofusin (Mfn) proteins regulate the biogenesis and maintenance of the mitochondrial network, and when inactivated, cause a failure in the mitochondrial architecture and decreases in oxidative capacity and glucose oxidation. Exercise increases muscle mitochondrial content, size, oxidative capacity and aerobic glucose oxidation. To address if Mfn proteins are implicated in these exercise-induced responses, we measured Mfn1 and Mfn2 mRNA levels, pre-, post-, 2 and 24 h post-exercise. Additionally, we measured the expression levels of transcriptional regulators that control mitochondrial biogenesis and functions, including PGC-1 $\alpha$ , NRF-1, NRF-2 and the recently implicated ERR $\alpha$ . We show that Mfn1, Mfn2, NRF-2 and COXIV mRNA were increased 24 h post-exercise, while PGC-1 $\alpha$  and ERR $\alpha$  mRNA increased 2 h post-exercise. Finally, using *in vitro* cellular assays, we demonstrate that Mfn2 gene expression is driven by a PGC-1 $\alpha$  programme dependent on ERR $\alpha$ . The PGC-1 $\alpha$ /ERR $\alpha$ -mediated induction of Mfn2 suggests a role of these two factors in mitochondrial fusion. Our results provide evidence that PGC-1 $\alpha$  not only mediates the increased expression of oxidative phosphorylation genes but also mediates alterations in mitochondrial architecture in response to aerobic exercise in humans.

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Peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) is a nuclear transcriptional coactivator that regulates several important metabolic processes, including mitochondrial biogenesis, adaptive thermogenesis, respiration, insulin secretion and gluconeogenesis (Knutti & Kralli, 2001; Puigserver & Spiegelman, 2003; Kelly & Scarpulla, 2004). PGC-1 $\alpha$  induces mitochondrial biogenesis by coactivating specific transcription factors, such as the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) (Wu *et al.* 1999) and the nuclear receptor oestrogen receptor alpha (ERR $\alpha$ ) (Mootha *et al.* 2004; Schreiber *et al.* 2004). The expression of PGC-1 $\alpha$  mRNA is prominent in tissues with high energy demands, such as heart and skeletal muscle (Knutti & Kralli, 2001; Puigserver & Spiegelman, 2003), and is induced in response to signals of metabolic stress, such as exercise (Goto *et al.* 2000; Baar *et al.* 2002; Pilegaard *et al.* 2003; Russell

*et al.* 2003). The activation and/or overexpression of PGC-1 $\alpha$  in cells and rodents regulates the expression of genes involved in mitochondrial biogenesis and oxidative phosphorylation (Wu *et al.* 1999; Lin *et al.* 2002; Mootha *et al.* 2004; Schreiber *et al.* 2004). Although an increase in human skeletal muscle PGC-1 $\alpha$  mRNA is associated with exercise it has yet to be established whether this is a causal relationship for the regulation of human muscle biogenesis.

The impairment of mitochondrial function has been suggested to be a crucial factor in the pathogenesis of insulin resistance (Kelley *et al.* 2002). PGC-1 $\alpha$  expression, as well as several other oxidative phosphorylation genes, have been claimed to be reduced in subjects with diabetes and insulin resistance, when compared with healthy subjects (Mootha *et al.* 2003; Patti *et al.* 2003). However, neither of these studies adequately controlled for physical activity levels making the interpretation of the data complex. Although reductions in PGC-1 $\alpha$  and PGC-1 $\alpha$ -dependent pathways seem plausible to contribute

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to the impaired bioenergetic capacity of skeletal muscle mitochondria observed in type II diabetes (Kelley *et al.* 2002; Mootha *et al.* 2003; Patti *et al.* 2003), further investigations are required.

Endurance exercise is used as an intervention for the treatment of type II diabetes because it increases mitochondrial content, size and skeletal muscle oxidative capacity (Holloszy & Coyle, 1984), as well as improves insulin sensitivity (Dela *et al.* 1992). The exercise-induced adaptations are likely to be due, in part, to an increase in PGC-1 $\alpha$  expression (Goto *et al.* 2000; Baar *et al.* 2002; Pilegaard *et al.* 2003; Russell *et al.* 2003; Short *et al.* 2003) and the effect of PGC-1 $\alpha$  on the aforementioned metabolic functions. Exercise is also reported to lead to the increased expression of some of the transcription factors that cooperate with PGC-1 $\alpha$ , such as NRF-1 and NRF-2 (Murakami *et al.* 1998; Baar *et al.* 2002; Short *et al.* 2003). The effects of exercise on the expression of ERR $\alpha$ , which was recently implicated in the process of mitochondrial biogenesis, have not yet been studied.

Skeletal muscle mitochondria from insulin resistant obese compared with healthy subjects also demonstrates a reduced gene and protein expression of mitofusin-2 (Mfn2), a key player involved in the formation and maintenance of the mitochondrial network (Bach *et al.* 2003). Inactivation of Mfn2 causes a dramatic failure in mitochondrial architecture, due to the lack of mitochondrial fusion, and leads to a significant decrease in oxidative capacity and glucose oxidation (Bach *et al.* 2003). Because exercise increases not only mitochondrial biogenesis but also oxidative capacity, we tested the hypothesis that exercise may also increase the expression of Mfn2 and/or of the related isoform, Mfn1.

We report here for the first time that acute exercise increases the expression of Mfn1, Mfn2 and ERR $\alpha$  mRNA in human skeletal muscle. We also demonstrate *in vitro* that Mfn2 can be induced directly by the cooperative actions of PGC-1 $\alpha$  and ERR $\alpha$ . Our findings suggest that the metabolic signals that are initiated by contracting skeletal muscle are capable of stimulating the transcription of genes involved not only in mitochondrial biogenesis, but also in mitochondrial fusion, possibly via a PGC-1 $\alpha$ /ERR $\alpha$ -dependent pathway.

## Methods

### Subject details

The subjects consisted of 11 trained male cyclists,  $36 \pm 4.9$  years of age (mean  $\pm$  s.d.), maximal oxygen consumption ( $\dot{V}_{O_{2,max}}$ )  $57.6 \pm 7$  ml kg<sup>-1</sup> min<sup>-1</sup>, mass  $75.3 \pm 9$  kg, body fat  $16.4 \pm 4.5\%$ . An additional five subjects, matched for age ( $34 \pm 7.2$  years) and mass ( $78.1 \pm 4$  kg), were included as a control group for the effect of time. The control group did not complete the

exercise trials but did undergo the muscle biopsies at the same time intervals as the exercising subjects. The entire study was approved by the local medical society ethical committee and all participants gave their informed consent and agreed to muscle biopsies and, where required, the physiological testing. The study conformed to the Declaration of Helsinki.

### Measurement of oxygen consumption

$\dot{V}_{O_{2,max}}$  was measured using a Quark B2 metabolic cart (Cosmed, Rome, Italy) while subjects were cycling on an ergometer (Ergoline 900, Sensor Medic, Bitz, Germany). The subjects began cycling at a power of 90 W. The power was increased by 30 W every 3 min until the subject could not maintain a minimal revolution of 75 r.p.m. At the end of each step, lactate concentration was obtained (Lactate Pro, Axon Lab, Baden, Switzerland). The duration of the test lasted between 20 and 30 min. Heart rate (Polar, LMT Leuenberger Medizintechnik, Wallisellen, Switzerland) and oxygen consumption were measured continually throughout the test.  $\dot{V}_{O_{2,max}}$  was calculated as the highest value averaged over a 30 second period (Russell *et al.* 2003).

### Fat free mass

Fat free mass was determined by plethysmographic measurement of body volume using the BOD POD Body Composition System (Life Measurement Instruments, Concord, USA) (Dempster & Aitkens, 1995). This device uses the relationship between pressure and volume to derive the body volume of a subject seated inside a fiberglass chamber.

### Experimental procedure

The subjects performed a 10 km cycling time trial that included an increase in altitude from 500 to 1250 m. This 10 km route was chosen because it was familiar to all of the subjects participating in the study. The time trial for all subjects was performed on the same day. The diet of all the participants was matched 24 h before and after the test. The liquid consumption during and after the test was restricted to water. Muscle biopsies and blood samples were obtained at four time points: a week before the exercise trial, immediately after exercise, and 2 and 24 h post-exercise.

### Muscle biopsy technique

Skeletal muscle samples were obtained under local anaesthesia (Rapidocaine, 1% plain) from the belly of the vastus lateralis muscle using a percutaneous needle biopsy technique (Pro-Mag, Medical Device Technologies Inc.,

Gainsville, FL, USA). An incision was made in the skin and three individual muscle samples were taken at each time point. The four muscle biopsies were taken from different incisions approximately 1.5–2.0 cm apart. The muscle samples were immediately frozen in liquid nitrogen and used for RNA (for samples for all four time points) and mitochondrial protein extraction (for samples pre- and 24 h post-exercise).

### Cell culture and infections

SAOS2 cells cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 9% fetal calf serum were infected on day 1 with adenoviruses expressing siRNA for ERR $\alpha$  or the control AdSUPER at a multiplicity of infection of 100, and on day 4 with adenoviral vectors expressing either green fluorescent protein (GFP) or PGC-1 $\alpha$  at a multiplicity of infection of 40. RNA and protein were isolated 24 h after the infection with GFP/PGC-1 $\alpha$  viruses. All vectors have been previously described (Schreiber *et al.* 2003).

### RNA extraction and real time quantitative PCR

RNA from skeletal muscle (approximately 15 mg of muscle) was extracted using a commercially available preparation, peqGOLD Tri-Fast (Peqlab, Germany). Three micrograms of RNA was reverse transcribed to cDNA using Random Hexamer primers and a Stratascript enzyme (Stratagene, the Netherlands). RNA from the SAOS2 cells was extracted using Trizol and reverse transcribed using a Superscript enzyme (Invitrogen, USA). Real-time PCR was performed using an MX3000p thermal cycler system and Brilliant SYBER Green QPCR Master Mix (Stratagene, the Netherlands). The PCR conditions for all genes consisted of one denaturing cycle at 90°C for 10 min, followed by 40 cycles consisting of denaturing at 90°C for 30 s, annealing at 60°C for 60 s and elongation at 72°C for 60 s. At the end of the PCR the samples were subjected to a melting curve analysis. To control for any variations due to efficiencies of the reverse transcription and PCR, 18S and acidic ribosomal phosphoprotein PO (36B4) were used as internal controls for the skeletal muscle and SAOS2 RNA, respectively. mRNA expression was calculated as follows. The number of cycles at which the best-fit line through the log-linear portion of each amplified curve intersects the noise band is inversely proportional to the log copy number (Higuchi *et al.* 1993). This value is referred to as the critical threshold ( $C_T$ ) value. The  $\Delta C_T$  was calculated by subtracting the  $C_T$  for 18S, or 36B4, from the  $C_T$  for the gene of interest. The relative expression of the gene of interest is calculated using the expression  $2^{-\Delta C_T}$  and reported as arbitrary units. All PCR runs were performed in triplicate. PCR primer sequences are provided in Table 1.

**Table 1. Primer sequences and annealing temperatures used for the PCR**

Gene	Sequence 5'–3'	Temp
Mfn1	sense TGT TTT GGT CGC AAA CTC TG	60
	Anti CTG TCT GCG TAC GTC TTC CA	
Mfn2	sense ATG CAT CCC CAC TTA AGC AC	60
	Anti CCA GAG GGC AGA ACT TTG TC	
PGC-1 $\alpha$	sense TCA GTC CTC ACT GGT GGA CA	62
	Anti TGC TTC GTC GTC AAA AAC AG	
ERR $\alpha$	Sense TTCTCATCGCTGTCGCTGTCT	64
	Anti CAGCCGCCGCACTAGTTG	
NRF-1	Sense GGT GCA GCA CCT TTG GAG AA	60
	Anti CCA GAG CAG ACT CCA GGT CTT C	
NRF-2	Sense CAA GAA CGC CTT GGG ATA CC	60
	Anti AAA CCA CCC AAT GCA GGA CTT	
COXIV	Sense CAT GTG GCA GAA GCA CTA TGT GT	60
	Anti GCC ACC CAC TCT TTG TCA AAG	
18S	Sense GAG GAT GAG GTG GAA CGT GT	60
	Anti GGA CCT GGC TGT ATT TTC CA	
36B4	Sense GTG ATG TGC AGC TGA TCA AGA CT	60
	Anti GAT GAC CAG CCC AAA GGA GA	

Mfn1, mitofusin-1; Mfn2, mitofusin-2; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$ ; ERR $\alpha$ , oestrogen related receptor- $\alpha$ ; NRF-1, nuclear respiratory factor-1; NRF-2, nuclear respiratory factor-2; COXIV, cytochrome c oxidase subunit IV; 18S, ribosomal 18S; 36B4, acidic ribosomal phosphoprotein PO.

### Mitochondrial extraction

Mitochondria were extracted from approximately 25 mg of skeletal muscle. The muscle was immersed in ice-cold medium buffer A (0.1 M KCl, 5 mM MgCl<sub>2</sub>, 5 mM EGTA, 5 mM sodium pyrophosphate, pH adjusted to 7.4, 2  $\mu$ M leupeptin, 2  $\mu$ M pepstatin, 0.5 mM phenylmethylsulphonylfluoride (PMSF) for 2  $\times$  10 min. Following this, the muscle was finely minced in 1/10 (w/v) buffer B (0.25 M saccharose, 50 mM KCl, 5 mM EDTA, 1 mM sodium pyrophosphate, 5 mM MgCl<sub>2</sub>, pH adjusted to 6.8, 2  $\mu$ M leupeptin, 2  $\mu$ M pepstatin, 0.5 mM PMSF) and then disrupted with a motor driven Teflon/glass homogenizer. The entire procedure was performed at 0–4°C. The homogenate was centrifuged at 1300 g for 10 min at 4°C, and the supernatant was removed and kept on ice (SN1). The pellet was resuspended with buffer B and centrifuged again at 1300 g for 10 min at 4°C. The second supernatant obtained (SN2) was mixed with the SN1 and centrifuged at 9000 g for 15 min at 4°C. The mitochondrial pellet was re-suspended in buffer B and protein concentration determined using a Micro BCA protein assay (Pierce, Rockford, IL, USA).

### Western blotting

Forty micrograms of muscle mitochondrial protein fractions were subjected to Western analysis using an

antibody raised against Mfn2 (Bach *et al.* 2003). Electrophoresis was performed using a 10% SDS PAGE gel in cold (4°C) buffer containing 25 mM Tris pH 8.8, 192 mM glycine and 20% methanol. After protein transfer, PVDF membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.05% Tween-20, and thereafter antibody incubation was performed with gentle shaking overnight at 4°C at a dilution of 1:400 in the 5% non-fat dry milk in 0.5% Tween-20/PBS. After the primary antibody incubation, the membranes were incubated at room temperature for 60 min with a horseradish peroxidase-conjugated goat antirabbit IgG (Cell Signalling, Beverly, MA, USA) at a dilution of 1:2000. The membranes were then washed for 4 × 5 min in 0.05% Tween-20/PBS and treated for 1 min with chemiluminescence substrate (Pierce, Rockford, IL, USA). Finally, X-ray film was exposed to the PVDF membranes for 1 min. Porin was used to control for the amount of protein loaded into each well (Sigma, Basel). The reaction product of each blot was analysed by densitometry using Scion imageware. SAOS2 protein extracts were prepared by lysing cells in 100 mM Tris pH 7.5, 1% NP40, 250 mM NaCl, 1 mM EDTA buffer. Extracts were then subjected to western analysis using antibodies against PGC-1 $\alpha$  (Schreiber *et al.* 2003) and ERR $\alpha$  (Johnston *et al.* 1997).

### Chromatin immunoprecipitation (ChIP)

SAOS2 cells ( $\sim 4.0 \times 10^6$ ) cultured in 15 cm plates were infected with GFP or PGC-1 $\alpha$  adenoviruses at a multiplicity of infection of 50. Twenty-four hours post-infection, cells were treated with 1% formaldehyde (cross-linker) for 7.5 min, harvested, and sonicated to  $\sim 500$  bp fragments, as described in (Chen *et al.* 1999). Sonicated chromatin was precleared for 2 h at 4°C with a mixture of protein A/protein G-sepharose (Amersham Biosciences, Uppsala Sweden). One-tenth of each sample was used to estimate input or 'total' DNA. The remaining 9/10 was split three ways and incubated overnight at 4°C with control (anti-GFP), anti-ERR $\alpha$  (generous gift of Dr V. Giguere; Sladek *et al.* 1997), or anti-PGC-1 $\alpha$  antibodies (Schreiber *et al.* 2003). After an additional incubation with protein A/protein G-sepharose for 2 h at 4°C, immunocomplexes were processed as previously described (Chen *et al.* 1999). DNA from each immunoprecipitation was then quantified with real-time PCR, using the Chromo4 system (Bio-Rad, Waltham, USA) and the following primers: 5'-AAATACAGCGGTGGATGTTAGAGA-3' and 5'-CCAGGCCTAGGTTGAAGTGA-3' for the Mfn2 gene; 5'-TTTCCAGCCCCCAATCTCA-3' and 5'-TCAGCGCCACCTGGTTCTT-3' for the Hsp70 gene. Genomic copy number was determined based on a

**Table 2. Subjects characteristics (exercising group)**

Variable	Mean $\pm$ s.d.
Age (years)	36 $\pm$ 4.9
Weight (kg)	75.3 $\pm$ 9.0
Body fat (%)	16.4 $\pm$ 4.5
$\dot{V}_{O_{2,max}}$ (ml kg <sup>-1</sup> min <sup>-1</sup> )	57.6 $\pm$ 7.0
$P_{max}$ (W kg <sup>-1</sup> )	4.4 $\pm$ 0.5
LT (ml kg <sup>-1</sup> min <sup>-1</sup> )	46.7 $\pm$ 7.5
10 km time (min)	46.2 $\pm$ 7.4

$P_{max}$ , maximal power output; LT, lactate threshold.

dilution series of sonicated SAOS2 genomic DNA. Values were normalized against 'total' DNA for each sample.

### Statistics

A one-way ANOVA with repeated measures, followed by linear contrasts, was used to compare the effects of exercise or time on the expression of Mfn1, Mfn2, PGC-1 $\alpha$ , ERR $\alpha$ , NRF-1, NRF-2 and COX IV. A one-way ANOVA, followed by a Tukey-Kramer *post hoc* test, was used to compare the expression of Mfn1 and Mfn2 in the different infected cells. The  $\alpha$  level of significance for the ANOVA was set at 0.05.

### Results

Characteristics of the subjects performing the exercise trial are presented in Table 2. The performance time of the 10 km cycling time trial was correlated with  $\dot{V}_{O_{2,max}}$  ( $r = -0.85$ ;  $P = 0.001$ ), lactate threshold (LT) ( $r = -0.85$ ;  $P = 0.001$ ) and maximal power output ( $P_{max}$ ) ( $r = -0.88$ ;  $P = 0.0001$ ), which were measured in the laboratory.  $\dot{V}_{O_{2,max}}$  and LT are indicators of physical fitness levels and strong predictors of performance (Grant *et al.* 1997).

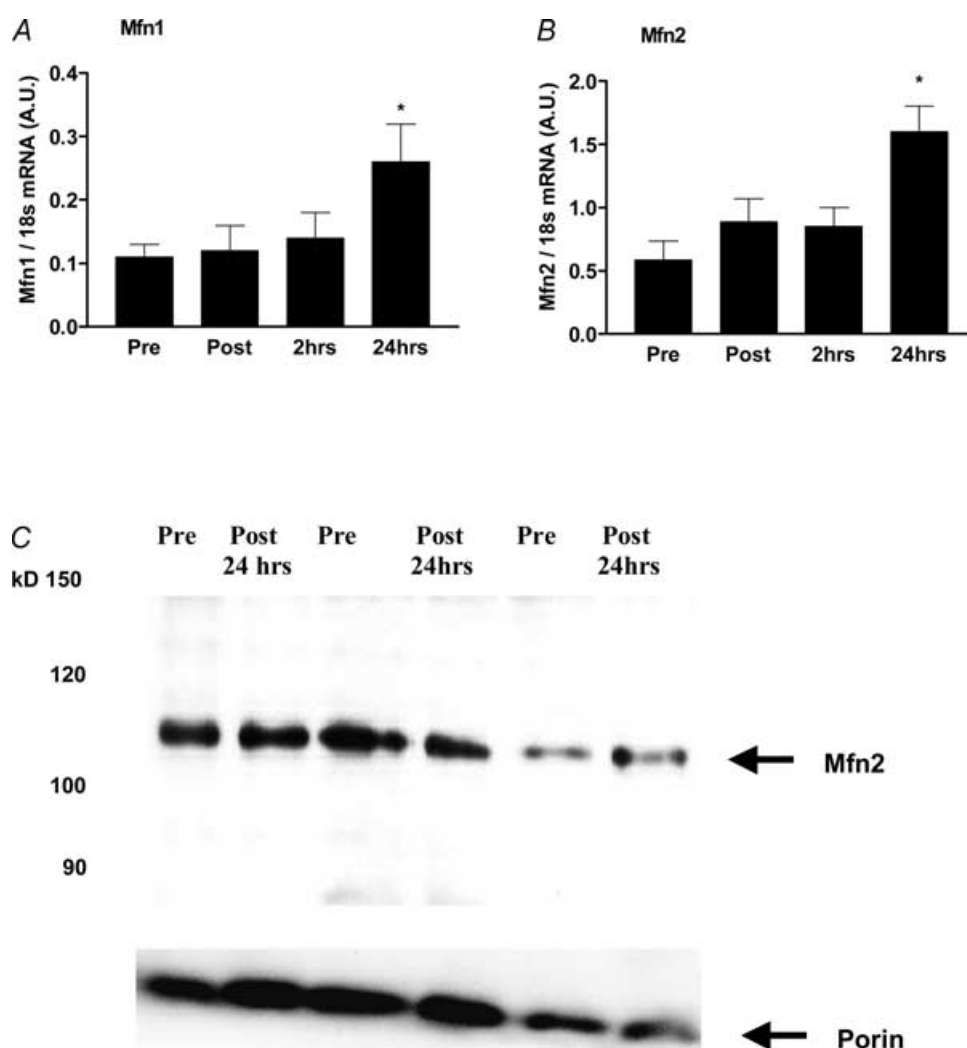
Although it is well known that exercise improves energy metabolism via the stimulation of mitochondrial biogenesis (Irrcher *et al.* 2003), it is unknown if exercise also regulates the genes involved in mitochondrial fusion. Using quantitative PCR, we show here that Mfn1 and Mfn2 mRNA levels were increased in human skeletal muscle by 2.4-fold and 2.7-fold, respectively ( $P < 0.05$ ), 24 h after an acute exercise bout (Fig. 1). We could not detect a change in the levels of Mfn2 protein (Fig. 1) at 24 h post-exercise. It is possible that the increases in the Mfn2 protein level are transient and not detectable after 24 h, or that the protein increases at a later time point.

PGC-1 $\alpha$  is an important player in mitochondrial biogenesis (Wu *et al.* 1999) and its expression has been shown to be increased in human muscle after exercise (Pilegaard *et al.* 2003; Russell *et al.* 2003; Short *et al.* 2003). In cell culture systems, PGC-1 $\alpha$  is reported to also

lead to the induction of some of the transcription factors with which it cooperates in mitochondrial biogenesis, such as NRF-1, NRF-2, and ERR $\alpha$  (Wu *et al.* 1999; Schreiber *et al.* 2003). To evaluate which of these transcriptional regulators are induced after a single bout of acute exercise in human muscle, we measured the mRNA levels of PGC-1 $\alpha$ , ERR $\alpha$ , NRF-1 and NRF-2. PGC-1 $\alpha$  and ERR $\alpha$  mRNA levels increased immediately after exercise, with a peak at 2 h post-exercise (3-fold increase at 2 h,  $P < 0.05$ ) (Fig. 2). NRF-1 levels did not change significantly at any of the times tested, while NRF-2 levels increased 2 h (3-fold;  $P < 0.05$ ) and 24 h post-exercise (5-fold;  $P < 0.01$ ) (Fig. 3). As PGC-1 $\alpha$  is also known to up-regulate COX IV (Wu *et al.* 1999), a nuclear encoded protein of the mitochondrial respiratory chain and a marker of mitochondrial biogenesis (Hood, 2001), we also measured

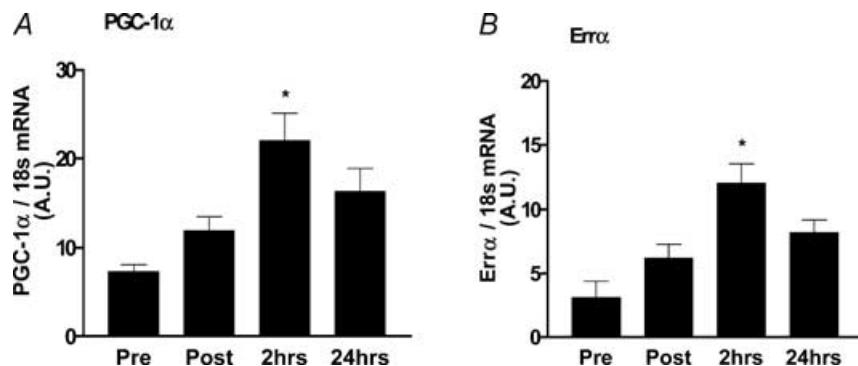
its mRNA expression. We observed a 2-fold increase in COX IV mRNA at 24 h post-exercise ( $P < 0.05$ ) (Fig. 3). To establish that the changes in gene expression were due to the exercise intervention and not due to a variation of time, a control group completed the muscle biopsy time course without exercise. As shown in Table 3 the expression of all genes measured was stable across the four biopsy sampling times demonstrating that the changes in gene expression observed in the present study was due to the exercise intervention employed.

Our *in vivo* results showed that exercise leads to increases in the gene expression of the mitochondrial fusion proteins Mfn1 and Mfn2, as well as that of PGC-1 $\alpha$  and its coactivators of mitochondrial biogenesis, ERR $\alpha$  and NRF-2. We next tested whether Mfn1 and Mfn2 could be regulated by PGC-1 $\alpha$  and/or ERR $\alpha$ , using an *in vitro*



**Figure 1.** The effect of acute exercise on Mfn1 mRNA (A), Mfn2 mRNA (B) and Mfn2 protein (C) levels measured pre-, post- and 2 and 24 h post-exercise

Mfn2 protein content was normalized against porin protein content. The blot shown is from 3 subjects representative of the 11 subjects used in the analysis. \* $P < 0.05$ , significantly different from pre-exercising levels.



**Figure 2.** The effect of acute exercise on PGC-1α (A) and ERRα (B) mRNA measured pre-, post-, and 2 and 24 h post-exercise. \* $P < 0.05$ , significantly different from pre exercising levels.

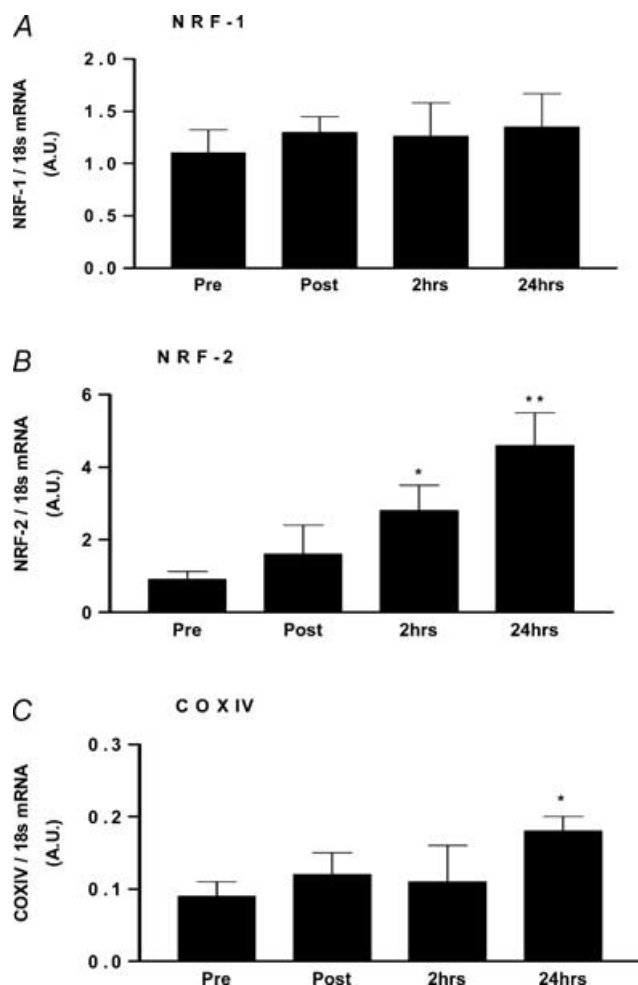
cell system. Expression of PGC-1α in SAOS2 cells leads to mitochondrial biogenesis, in a manner that depends on endogenous ERRα, and which is similar to PGC-1α function in a muscle cell line (Mootha *et al.* 2004; Schreiber

*et al.* 2004). Expression of PGC-1α in SAOS2 cells also led to the induction of Mfn2, but not Mfn1 expression (Fig. 4). The PGC-1α-driven induction of Mfn2 was mediated by the endogenous ERRα, since it was not seen in cells where ERRα expression was inhibited by small interfering (si) RNA (see Fig. 4C for ERRα protein levels). These results suggested that ERRα enables the induction of Mfn2 by PGC-1α, and that PGC-1α and ERRα are not sufficient for the induction of Mfn1. Mfn1 regulation may require a different PGC-1α controlled pathway that is not active in the SAOS2 cells, or a distinct exercise-induced stimulus that is independent of PGC-1α.

To test if ERRα and PGC-1α act directly at the Mfn2 gene to induce its mRNA expression we asked whether ERRα and PGC-1α interact physically with the Mfn2 promoter. Analysis of the Mfn2 promoter sequence indicated two regions with putative ERRα/nuclear receptor binding sites; the -421/-397 sites in particular are highly conserved in the mouse (top) and human (bottom) genes (Fig. 5A). Using chromatin immunoprecipitation assays, we detected specific binding of both PGC-1α and ERRα at the Mfn2 promoter in SAOS2 cells expressing PGC-1α, but not in the control GFP-expressing cells.

## Discussion

A major adaptation to exercise is the improvement in skeletal muscle mitochondrial function and capacity, which are important determinants of insulin sensitivity. Improvements in mitochondrial function require an increase in the proteins that participate in the generation of a dynamic mitochondrial network. We show in this study that, in response to acute endurance exercise, there is an increase in the gene expression of the mitochondrial fusion proteins Mfn1 and Mfn2, in human skeletal muscle. Additionally, there is an increase in the mRNA levels of the transcriptional coactivator PGC-1α and the PGC-1α-interacting transcription factors ERRα and NRF-2. Notably, this is the first study to report changes in ERRα mRNA levels following exercise. Furthermore, there is an increase in COX IV mRNA, a PGC-1α target gene and marker of mitochondrial biogenesis. We have also show



**Figure 3.** The effect of acute exercise on NRF-1 (A), NRF-2 (B) and COX IV (C) mRNA levels measured pre-, post-, and 2 and 24 h post-exercise.

\* $P < 0.05$ , significantly different from pre exercising levels;

\*\* $P < 0.01$ , significantly different from all other time points.

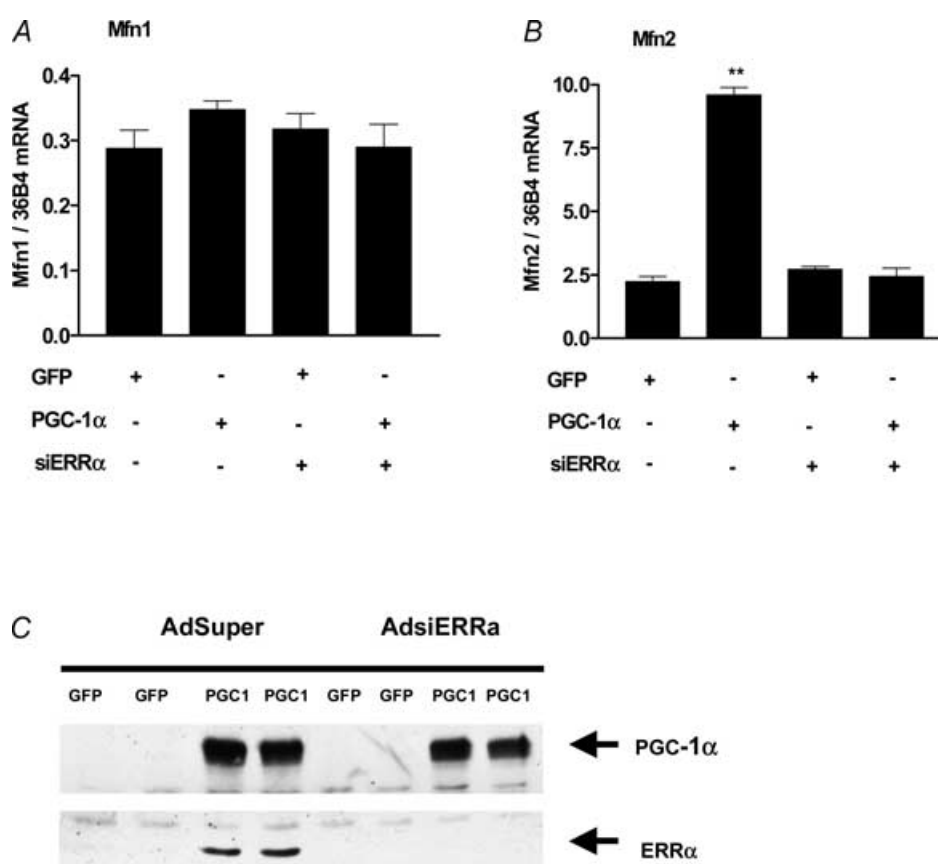
**Table 3.** Gene expression values from the non-exercising control group

Gene	Pre	Post	2 h	24 h
Mfn1	0.09 $\pm$ 0.03	0.11 $\pm$ 0.06	0.10 $\pm$ 0.09	0.11 $\pm$ 0.06
Mfn2	0.70 $\pm$ 0.12	0.62 $\pm$ 0.21	0.83 $\pm$ 0.17	0.84 $\pm$ 0.223
PGC-1 $\alpha$	7.67 $\pm$ 0.9	8.21 $\pm$ 1.23	8.66 $\pm$ 0.6	7.00 $\pm$ 0.92
ERR $\alpha$	3.76 $\pm$ 0.22	3.59 $\pm$ 0.34	3.99 $\pm$ 0.51	3.55 $\pm$ 0.22
NRF-1	1.40 $\pm$ 0.38	1.00 $\pm$ 0.22	1.20 $\pm$ 0.32	1.86 $\pm$ 0.25
NRF-2	1.23 $\pm$ 0.62	1.77 $\pm$ 0.66	2.06 $\pm$ 0.52	1.99 $\pm$ 0.49
COXIV	0.11 $\pm$ 0.06	0.09 $\pm$ 0.07	0.13 $\pm$ 0.04	0.12 $\pm$ 0.01

The headings Pre, Post, 2 h and 24 h are with respect to the time intervals between biopsies and correspond to the time intervals for the exercising group. Mfn1, mitofusin-1; Mfn2, mitofusin-2; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$ ; ERR $\alpha$ , oestrogen related receptor- $\alpha$ ; NRF-1, nuclear respiratory factor-1; NRF-2, nuclear respiratory factor-2; COXIV, cytochrome c oxidase subunit IV.

that PGC-1 $\alpha$  and ERR $\alpha$  recognize the Mfn2 promoter and induce Mfn2 expression in cultured cells, suggesting that PGC-1 $\alpha$  and ERR $\alpha$  mediate the Mfn2 induction by exercise *in vivo*.

Previous studies in HeLa cells have shown that Mfn1 and Mfn2 are essential for the maintenance of mitochondrial morphology, and that the two mitofusins function co-operatively in mitochondria morphogenesis (Eura *et al.* 2003). Knock down of Mfn2 in L6E9 myotubes results in a dramatic discontinuity of the mitochondrial network (Bach *et al.* 2003). Mfn proteins also appear to play a major role in mitochondrial metabolism; the repression of Mfn2 in L6E9 myotubes and 10T1/2 cells reduces glucose oxidation by 30 and 70%, respectively, and oxygen consumption by 30% (Bach *et al.* 2003). In line with these observations, obese humans, who demonstrate a lower insulin-stimulated glucose disposal and a reduced oxidative capacity, also have 36 and 43%, respectively, lower skeletal muscle Mfn2 mRNA and protein expression (Bach *et al.* 2003). Exercise improves mitochondrial function in both insulin resistant and healthy subjects by increasing mitochondrial oxidative metabolism. Our results suggest that this adaptation may be, in part, a response to increases in Mfn1 and Mfn2. Although we did not detect an increase in Mfn2 protein content at 24 h post-exercise it is still possible that Mfn2 protein levels

**Figure 4.** PGC-1 $\alpha$  and ERR $\alpha$  induce Mfn2

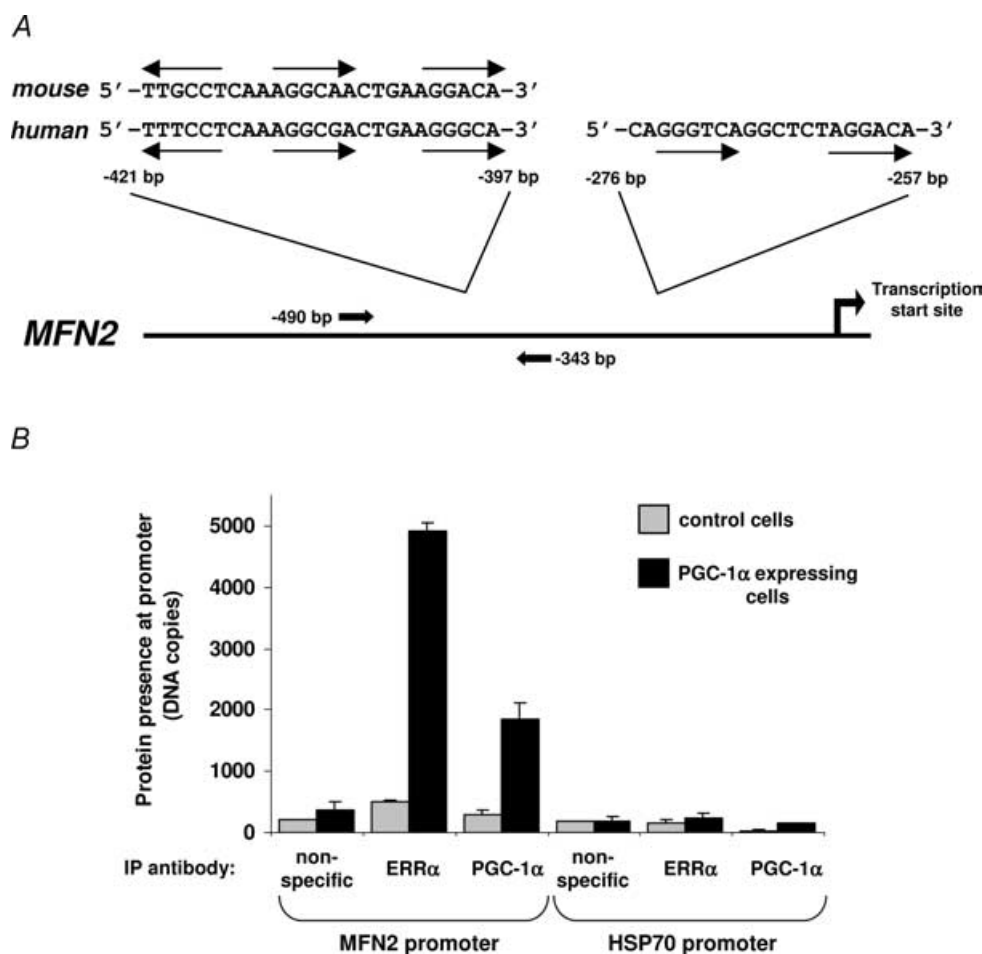
Mfn1 (A), and Mfn2 (B) mRNA levels were determined 24 h after infection of SAOS2 cells with GFP/PGC-1 $\alpha$  viruses.

\*\* $P < 0.01$ , significantly different from other infected cells. C, Western blot showing the effect of the siRNA for ERR $\alpha$  on the expression (or levels) of ERR $\alpha$  protein. Each example is shown in duplicate.

either rise transiently or with considerably slower kinetics than the RNA.

PGC-1 $\alpha$  has been shown to increase in response to exercise in human (Pilegaard *et al.* 2003; Russell *et al.* 2003; Short *et al.* 2003; Norrbom *et al.* 2004) and rodent skeletal muscle (Goto *et al.* 2000; Baar *et al.* 2002; Terada *et al.* 2002). PGC-1 $\alpha$  is a major player in mitochondrial biogenesis, an adaptation stimulated by exercise, so it would seem likely that PGC-1 $\alpha$  is involved in exercise-induced mitochondrial biogenesis. In addition to the role of PGC-1 $\alpha$  as a regulator of mitochondrial biogenesis, we demonstrated that PGC-1 $\alpha$ -activated programmes are also involved in mitochondrial fusion. PGC-1 $\alpha$  can activate several

pathways regulating mitochondrial biogenesis. Recently, it was demonstrated that PGC-1 $\alpha$  interacts with and activates the transcriptional function of ERR $\alpha$  (Huss *et al.* 2002; Schreiber *et al.* 2003); this activation then leads to the induction of ERR $\alpha$  target genes (including ERR $\alpha$  itself), many of which are important for mitochondrial biogenesis (Mootha *et al.* 2004; Schreiber *et al.* 2004). Our *in vivo* results presented here show that, during recovery from acute exercise, there is an increase in both PGC-1 $\alpha$  and ERR $\alpha$  mRNA, suggesting that this is an important pathway for the exercise-stimulated mitochondrial biogenesis and mitochondrial function observed in human skeletal muscle. The fast induction of ERR $\alpha$ , parallel to that of PGC-1 $\alpha$ , is consistent with ERR $\alpha$



**Figure 5. ERR $\alpha$  and PGC-1 $\alpha$  bind to sequences 5' of the MFN2 promoter in SAOS2 cells**

A, diagram of the promoter and 5' sequences of the human MFN2 gene. Two regions with putative ERR $\alpha$ /nuclear receptor binding sites are detailed above the MFN2 diagram; the -421/-397 sites are conserved in the mouse (top) and human (bottom) genes, as indicated. Arrows below the sequence depict putative recognition half-sites for nuclear receptors (consensus being AGGTCA). Numbering is relative to the MFN2 transcription start site. Also indicated are the positions of the oligonucleotides used to amplify the -343/-490 region. B, chromatin immunoprecipitation from SAOS2 cells after infection with GFP- (light grey) or PGC-1 $\alpha$ - (dark grey) expressing adenoviruses. Quantitative PCR assays were used to detect the presence of the -343/-490 region of the Mfn2 promoter or, as control, the HSP70 promoter in DNA immunoprecipitated with the indicated antibodies (for 'non-specific', samples were immunoprecipitated with an antibody against GFP). Results shown are the mean  $\pm$  s.e.m. from two of four representative independent experiments.

being an early direct target of PGC1, as shown in studies in cultured cells (Schreiber *et al.* 2003; Laganieri *et al.* 2004).

Another PGC-1 $\alpha$  coactivated circuit that regulates mitochondrial biogenesis includes the nuclear respiratory factors NRF-1 and NRF-2 (Wu *et al.* 1999). These NRFs activate genes encoding proteins of the respiratory chain, such as  $\beta$ -ATP synthase, COX IV and CytC (Virbasius & Scarpulla, 1991; Kelly & Scarpulla, 2004), which are also targets of PGC-1 $\alpha$ /ERR $\alpha$  coactivation (Schreiber *et al.* 2004). In rodents, NRF-1 and NRF-2 (Murakami *et al.* 1998; Baar *et al.* 2002) are up-regulated 6–18 h after a single exercise bout, parallel to increases in PGC-1 $\alpha$  (Baar *et al.* 2002). In humans, NRF-1 and NRF-2 expression does not change after 3 h of single leg exercise nor after 4 weeks of single leg exercise training (Pilegaard *et al.* 2003). Additionally, NRF-1 did not change after 45 min (Norrbom *et al.* 2004) or 80 min of exhaustive single leg exercise nor after 4 h of whole body cycling exercise (Pilegaard *et al.* 2003). It has therefore been suggested that the basal level of NRF-1 in human skeletal muscle, if involved in acute exercise stimulated gene transcription, is sufficient to meet the needs of the cells (Pilegaard *et al.* 2003). In contrast, 16 weeks of whole body cycling exercise resulted in a small but significant 15% increase in NRF-1 mRNA in human skeletal muscle (Short *et al.* 2003). Our results show that acute whole body endurance exercise, results in an increase in NRF-2, but not NRF-1, at 2 and at 24 h post-exercise. With respect to exercise, the extent to which NRF-1 and NRF-2 are regulated at the level of expression may depend on the species, the specific muscle, and/or the type of exercise.

In the present study, the increase in PGC-1 $\alpha$ , ERR $\alpha$  and NRF-2 was also associated with an increase in COXIV at 24 h post-exercise. From our *in vivo* results it is difficult to ascertain whether the exercise induced increase in COX IV is due to a PGC-1 $\alpha$  coactivation of ERR $\alpha$ , NRF-1, NRF-2 or a combination of these factors. *In vitro* results from cellular assays have shown that COXIV induction by PGC-1 $\alpha$  depends, at least partly, on ERR $\alpha$  function (Schreiber *et al.* 2004). Additionally, it has been demonstrated in mouse C2C12 myotubes that PGC-1 $\alpha$  increases the transcriptional activity of both ERR $\alpha$  and the GA-repeat binding protein (GABP, the mouse homologue of NRF-2), forming a double-positive-feedback loop with PGC-1 $\alpha$  and driving the early expression of mitochondrial genes (Mootha *et al.* 2004). Whether NRF-1 is downstream of ERR $\alpha$  and NRF-2 in human skeletal muscle, as seems to be the case in the C2C12 cells (Mootha, 2004), is unknown. Possibly, the effects of long-term exercise on human skeletal muscle COXIV mRNA regulation (Vogt *et al.* 2001; Russell *et al.* 2003; Short *et al.* 2003) are synergistically controlled by PGC-1 $\alpha$ , ERR $\alpha$ , NRF-1 and NRF-2 pathways.

In summary, we have shown that acute endurance exercise induces the expression of Mfn1, Mfn2 and

ERR $\alpha$  mRNA levels in human skeletal muscle, during the 24 h post-exercise. We have also demonstrated *in vitro* that Mfn2, but not Mfn1, is induced directly by the transcriptional couple of PGC-1 $\alpha$  and ERR $\alpha$ . The PGC-1 $\alpha$ /ERR $\alpha$ -mediated induction of Mfn2 implicates PGC-1 $\alpha$  in mitochondrial fusion and adds yet another key metabolic function to its regulatory capabilities. Our results also suggest that exercise is not only responsible for providing signals that effect mitochondrial biogenesis, but also for promoting mitochondrial fusion, demonstrating another mechanism, targeted by exercise, which may assist in reducing insulin resistance.

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